REMARKS

I. Status of the Claims

Claims 1-5 and 7-18 are pending in the application, are under examination, and stand rejected, variously, under 35 U.S.C. §112, second paragraph, 35 U.S.C. §103, and for alleged obviousness-type double-patenting. The specific grounds for rejection, and applicants' response thereto, are set out in detail below.

II. Objection

Claim 4 is objected to for the use of the term "directed." Applicants point the examiner to the definition of "directed immobilisation" at page 8 of the specification for an explanation of this term. Reconsideration and withdrawal of the objection is respectfully requested.

III. Rejections Under 35 U.S.C. §112, Second Paragraph

Claims 1-5 and 7-12 and 14-17 remain rejected as indefinite. Applicants traverse, but in the interest of advancing the prosecution, the claims have been amended to recite that the bacteriophage tail protein binds to the core region of endotoxin, which defines that "similar" means at least the retention of the p12 phage tail protein function. Support for the amendment derives from, at least, page 11, lines 26-28, of the specification and is further enabled by the assay described in Example 12 and FIGS. 6C and 7, and the discussion of endotoxin core structure at page 1 of the specification.

Reconsideration and withdrawal of each of the foregoing rejections is respectfully requested.

IV. Rejection Under 35 U.S.C. §103

Claims 1-4, 11 and 15 are rejected as obvious over Suzuki *et al.* It is argued that one of skill in the art would have substituted the various disclosed buffer systems to arrive at the invention as now claimed, thus, given the broad interpetation of "p12-similar," the claims are obvious in view of the reference. Applicants again traverse.

First, applicants Suzuki *et al.* neither teach the detection of endotoxin nor the removal of endotoxin as presently claimed. The intent of their work was "to prepare H protein using genetic engineering techniques and examine whether H protein could, by itself, recognize the receptor LPS" (Suzuki *et al.*, p. 96, 2nd paragraph). At that time, a direct interaction between H protein and LPS as the host receptor for phiX174 was not shown (see Suzuki *et al.*, page 96, 1st paragraph). The conclusion suggests the need for further studies to combine H protein with F and G proteins to differentiate the recognition stage of the phage from the inactivation stage. Thus, regardless of whether "p12-similar" is interpreted in a broad or narrow fashion, one would not have been motivated to select H protein of phiX174 for the detection and/or removal of endotoxin.

Second, phiX174 is a phage that is specific for "rough mutants" (Suzuki *et al.*, page 95, introduction; page 98, right column). These are special mutants with a shortened version of LPS (no O-antigen) often found in less pathogenic laboratory bacterial strains, but the common and more pathogenic bacterial strains exhibit "smooth LPS," which is not even recognized by the H protein of phiX174 (Suzuki *et al.*, page 98, right column). Teaching of the reduced pathogenicity of rough strains due to the lack of long chains of repeating oligosaccharide chains is found in the introduction of Dlabac *et al.* (1997) (enclosed). Again, it would therefore not be obvious for the skilled artisan to select select H protein of phiX174 to remove or detect endotoxin.

Further differences between various specific embodiments of the claimed invention and Suzuki *et al.* are as follows. First, Suzuki *et al.* does not use the His-tag as coupling group like as in present claim 11, but only as vehicle to facilitate protein purification (Suzuki *et al.*, page 97, 1st paragraph). The authors even tried to remove the His-tag by protease treatment after purification (page 97, 2nd paragraph). Also, the phage protein was merely adsorbed to the microtiter plates, and not immobilized by coupling groups as in present claim 11. Finally, the LPS-labeled with biotin is directly detected in Suzuki *et al.* via a streptavidin-peroxidase complex (pages 97-98), whereas the "marked endotoxin" of present claim 15 is used as tool to detect the sample endotoxin to be detected in a competitive assay.

Based on the preceding, applicants respectfully submit that the claims, regardless of the definition of "p12-similar," would not have been rendered obvious by the teachings of Suzuki *et al.* Reconsideration and withdrawal of the rejection is therefore respectfully requested.

V. Double-Patenting

The examiner has maintained the provisional, non-statutory double-patenting rejection over the '415 application. Because the first allowed case should be passed to issue prior to any rejections being maintained, applicants thus need not address those rejections until at least one of the two applications is allowed.

VI. Conclusion

In light of the foregoing, applicants respectfully submit that all claims are in condition for allowance, and an early notification to that effect is earnestly solicited. The examiner is invited to contact the undersigned attorney at 512-536-3184 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,

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Pathogenicity and Protective Effect of Rough Mutants of *Salmonella* Species in Germ-Free Piglets

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In this study, two stable, rough, streptomycin-sensitive *Salmonella* mutants with different types of genetic defects were used to colonize groups of germ-free (GF) piglets. The lipopolysaccharide (LPS) of *Salmonella typhimurium* SF 1591 was of the Ra chemotype (complete core), whereas the LPS of the *S. minnesota* mR 595 deep-rough mutant contained only lipid A and 2-keto-3-deoxyoctulosonic acid (Re chemotype). Both strains readily colonized the intestinal tracts of GF piglets and were stable during the whole experiment. All animals survived, and only transient fever was observed in some piglets colonized with the SF 1591 strain. Finally, streptomycin and virulent, smooth, streptomycin-resistant *S. typhimurium* LT2 were administered perorally 1 week later. All piglets colonized previously with the deep-rough mutant mR 595 died of sepsis, in contrast to piglets infected with the LT2 strain and colonized with the SF 1591 mutant, all of which survived. This difference is explained by the penetration of the mesenteric lymph nodes, spleen, and liver by great numbers of live bacteria in the latter case, resulting in prominent systemic and local immune responses. On the other hand, live bacteria were found only rarely in the mesenteric lymph nodes of animals colonized with the mR 595 strain and a negligible antibody response was observed.

Germ-free (GF) piglets represent a unique experimental model of infection studies for two reasons. First, newborn piglets lack maternal antibodies and only traces of immunoglobulins produced prenatally are present in their sera (7, 41). Second, the endogenous intestinal microflora limiting bacterial multiplication in the gut lumen of infected conventional animals (43) is absent. *Escherichia coli* and *Salmonella* microorganisms multiply freely in the guts of GF piglets, reaching a maximum concentration within a few days (10). This fact is especially important in the case of pathogenic strains of these bacteria because the resulting "infectious dose" is several orders of magnitude higher than the dose applied and incomparable to the counts reached in conventional animals. Consequently, peroral infection of GF piglets with pathogenic strains of these bacteria leads to sepsis within a few hours and finally to death within 1 or 2 days (10, 37). On the other hand, colonization with some strains of *E. coli* does not evoke symptoms of clinical disease and histological examination of the intestinal mucosa shows only signs of "physiological inflammation" (1). However, even in such a case, numerous bacteria can be found in the mesenteric lymph nodes, spleen, and liver in the first week after colonization and in decreasing numbers for some weeks thereafter (40).

It is well known that rough *Salmonella* strains devoid of long chains of repeating oligosaccharide units (characteristic of the lipopolysaccharide [LPS] of smooth strains) showed highly reduced pathogenicity in conventional piglets (11, 38). However, GF piglets infected with spontaneous rough *Salmonella typhimurium* mutants of different chemotypes experience high levels of mortality (11). An explanation of this fact is that intestinal bacteria reverted to the smooth strain. Surprisingly, appreciable numbers of piglets in all groups survived despite the presence of pure cultures of smooth virulent *S. typhimurium* bacteria in their guts (10). These results suggest that rough *Salmonella* strains induced a protective immune response against virulent smooth strains.

To test this hypothesis, two stable, rough *Salmonella* mutants with different LPS chemotypes were used in this study. Both strains were stable under in vivo conditions, and GF piglets survived peroral application of them. Furthermore, despite the lack of O-specific polysaccharide, one of these rough mutants elicited a prominent protective effect against subsequent infection with the virulent *Salmonella* strain.

The work described here shows the differences between these two rough mutants in penetration, antibody response, and protective effect. These results are discussed with the aim to explain the mechanism of protection involved. In an accompanying study (42), the cellular changes in Peyer's patches and intestinal mucosae of these gnotobiotic animals were investigated.

MATERIALS AND METHODS

***Salmonella* strains.** A streptomycin-resistant mutant of the original streptomycin-sensitive strain *S. typhimurium* LT2 was obtained by plating the wild-type strain (10^{10} bacteria per plate) on meat-peptone agar containing streptomycin at 200 μg/ml. Both agglutinability by specific anti-O serum (4, 5, 12) and the pathogenicity of the selected mutant for mice and GF piglets were comparable to those of the original strain.

Salmonella strain SF 1591 is a stable chemotype Ra mutant of *S. typhimurium* M206 with a deletion in the His locus, the LPS of which has a complete core polysaccharide.

Salmonella strain mR 595 is a deep-rough mutant of *S. minnesota* (Re chemotype). Both of these strains are sensitive to streptomycin. The original wild-type strain and both rough mutants were obtained from O. Lüderitz (Max-Planck-Institut für Immunbiologie, Freiburg im Breisgau, Germany). The LPS structures of these strains were previously published (26, 27).

LPSs. The LPS of wild-type *S. typhimurium* LT2 was isolated by the phenol-water method and further purified by repeated ultracentrifugation at 105,000 × *g* for 4 h (44). LPSs of rough strains were extracted by the phenol-chloroform-petroleum ether (PCP) method of Galanos et al. (16). If needed, the latter method was also used to repurify the smooth LPS isolated by the former method to remove contaminating rough, incomplete LPS (36).

Animals. Miniature pigs of the Minnesota breed were used. Piglets were delivered by hysterectomy under halothane-oxygen anesthesia into sterile isolators and kept in GF conditions. They were fed a milk diet supplemented with vitamins (29). One-week-old GF piglets were colonized perorally with 5×10^8 bacteria of a given rough strain. One week later, a suspension containing 5×10^8

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streptomycin-resistant *S. typhimurium* LT2 bacteria was applied perorally. This method proved to be 100% effective in completely changing the streptomycin-sensitive population of rough strains in the intestine to the streptomycin-resistant, smooth strain of *S. typhimurium*. The stability of rough mutants in the gut and the effectiveness of subsequent colonization by the smooth strain were frequently monitored by agglutination of at least 50 colonies isolated at various intervals from the stool of individual piglets by using specific agglutinating antisera prepared by repeated intravenous immunization of rabbits with suspensions of heat-killed (1 h at 100°C) bacteria (24). A similar procedure was used for identification of bacteria isolated from mesenteric lymph nodes and other organs.

Other stool samples were cleared by repeated, brief, low-speed centrifugations with PBS (phosphate-buffered saline; pH 7.3) to remove gross particles. Smears of supernatants containing bacteria were prepared on slides, air dried, briefly fixed with acetone, and stored at −20°C. Immunoglobulin coating bacteria were detected by the indirect immunofluorescence method using mouse monoclonal antibodies (MAbs) against immunoglobulin G (IgG) and IgA (the MCA635, MCA636, and MCA638 clones purchased from Serotec, Oxford, United Kingdom) and IgM (MAb LiG4 kindly given by P. Dvořák, Brno, Czech Republic). The immunofluorescent reagent SwaMFITC was purchased from Medicamenta (Vysoké Mýto, Czech Republic).

Blood was obtained by heart puncture, and serum samples were stored at −20°C. Piglets were sacrificed by exsanguination under anesthesia, and samples of blood, spleen, mesenteric lymph nodes, and intestinal contents were taken for estimation of bacteria counts. Ileal contents were washed out with 5 ml of PBS and centrifuged. The supernatants were stored at −20°C for enzyme-linked immunosorbent assay (ELISA).

Detection of antibodies in sera and intestinal washings by ELISA. Antibody response was estimated in sera and intestinal washings by using purified LPS of smooth *S. typhimurium* LT2 and rough LPS of the Ra and Re chemotypes, respectively. Antigens suspended in distilled water at a concentration of 1 mg/ml were solubilized by triethylamine (1 μl/ml) and diluted to a final concentration of 100 μg/ml with PBS before transfer to the wells (10 μl/well) of Immulon (Dynatech) microplates. After overnight incubation at 4°C, the plates were thoroughly washed with PBS and blocked with a 1% solution of bovine serum albumin for 30 min at room temperature. Suitably diluted piglet sera or washings (repeatedly washed with PBS) were added and incubated for 2 h at 37°C. Pig hyperimmune antisera diluted 1:100 and newborn piglet sera diluted 1:5 were used as positive and negative controls. These controls were used to test the reproducibility of the system. The extinction of negative controls was below 0.05, and that of pig hyperimmune antisera was 0.3 or higher. After repeated washings with PBS containing 0.05% Tween 20 and a final washing with PBS, the bound antibodies were treated with 100 μl of peroxidase-conjugated rabbit anti-pig immunoglobulin diluted 1:1,000 (RaSwPx; Medicamenta) for 45 min at room temperature. After repeated washings with 0.05% Tween 20–PBS, the enzyme reaction was developed with 5 mM *o*-phenylenediamine in 100 mM phosphate buffer (pH 6) containing 2.5 mM H_2O_2 (50 μl/well). The reaction was stopped by adding 30 μl of 1 M H_2SO_4. The optical density was read on a Dynatech micro-ELISA reader at 492 nm. Immunoglobulin isotypes were detected by a sandwich technique using MAbs against porcine immunoglobulin isotypes as described elsewhere (7). The dilution of a sample was evaluated as positive when the extinction was 0.15 or higher.

SDS-polyacrylamide gel electrophoresis and Western blot analysis. The suspensions of LPS in distilled water were solubilized with triethylamine (2 μl/ml) and boiled for 5 min in fivefold-concentrated sodium dodecyl sulfate (SDS) buffer, and 2 μg/10 μl was applied and fractionated on an SDS–12% polyacrylamide gel for 3 h at 40 mA with a Mini Protean II apparatus (Bio-Rad Laboratories, Richmond, Calif.). When the tracking dye was near the rim of the gel, it was immediately transferred to a nitrocellulose membrane (Hybond-C-pure; Amersham) and blotting was performed at 200 mA for 1.5 h with the same apparatus. To block unbound sites, the nitrocellulose sheet was soaked in PBS containing 0.05% Tween 20 and 3% skim milk overnight at 4°C. After repeated washings in PBS-Tween 20 and distilled water, the sheets were air dried. Individual lanes were then cut with scissors and treated with piglet or hyperimmune rabbit antisera diluted 1:10 and 1:200 in PBS, respectively, with continuous gentle shaking for 1 h at room temperature. After three washings with PBS-Tween 20, RaSwFx (peroxidase-conjugated rabbit anti-pig immunoglobulin) diluted 1:1,000 with PBS was added to the blots treated with piglet sera and the mixture was incubated for 1 h at room temperature, while blots incubated with hyperimmune antisera were similarly treated with SwaRPx (peroxidase-conjugated porcine anti-rabbit immunoglobulin purchased from Medicamenta) diluted 1:1,000. After repeated washings with PBS-Tween 20 and one washing with Tris-buffered saline (pH 7.5), the enzymatic reaction was developed in a fresh solution of the same buffer containing 4-chloro-1-naphthol (30 μM) and H_2O_2. The reaction was stopped by adding distilled water.

RESULTS

Pathogenicity of rough *Salmonella* strains for GF piglets. Within 10 to 12 h after peroral application of *S. typhimurium* SF 1591 and *S. minnesota* mR 595 rough mutants, bacteria could be detected in the stool and constant concentrations were reached within 1 or 2 days. No difference in intestinal bacterial counts was observed between these two groups of animals. Bacterial counts increased from approximately 10^4 in the duodenal and jejunal contents to 10^{11} in the colon. The total numbers of living bacteria in the whole intestinal contents of 2-week-old minipigs were roughly estimated to be on the order of 10^{12}. A total of 100 colonies isolated from the stool of either group of animals during this period revealed no antigenic change when tested by slide agglutination using specific antisera.

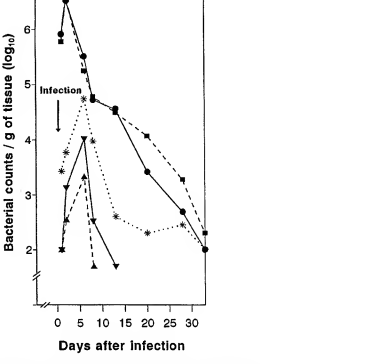

FIG. 1. Changes of bacterial counts in the spleen (▼), the liver (▲), and the mesenteric lymph nodes of the jejunum (✱), ileum (●), and colon (■) after colonization of GF piglets with *S. typhimurium* SF 1591. Each point is the geometric mean of estimates obtained from at least two piglets.

TABLE 1. Bacterial counts in the blood, spleen, liver, and mesenteric lymph nodes of piglets 1 week after infection with rough *Salmonella* strains

Tissue or fluid	Bacterial count[a] in indicated GF piglet infected with strain:			
	SF 1591 (Ra)		mR 595 (Re)	
	4/681	3/687	1/681	2/688
Blood	6.6×10^2	4.0×10^2	0	0
Spleen	1.4×10^4	2.9×10^3	0	0
Liver	4.0×10^2	3.4×10^3	0	0
MLN[b]	1.4×10^5	1.1×10^4	0	2.3×10^5
Intestine	NT	4.4×10^8	NT	5.6×10^9

[a] Values represent counts per gram of tissue or milliliter of fluid. NT, not tested.
[b] MLN, mesenteric lymph nodes.

TABLE 2. Bacterial counts in blood, spleen, liver, and mesenteric lymph nodes of piglets[a] infected with strain SF 1591 (Ra) and reinfected with virulent smooth strain *S. typhimurium* LT2

Tissue or fluid	Bacterial count[b] in piglet:				
	3/681	5/681	6/681	1/687	2/687
Blood	0	2.5×10^4	0	1.0×10^2	0
Spleen	0	4.1×10^3	0	2.3×10^4	1.4×10^4
Liver	0	1.5×10^3	0	2.7×10^4	0
MLN[c]	1.6×10^3	5.8×10^3	2.1×10^3	1.2×10^4	6.5×10^4

[a] All piglets were clinically healthy.
[b] Values represent counts per gram of tissue or milliliter of fluid.
[c] MLN, mesenteric lymph nodes.

Bacteria were detected in the mesenteric lymph nodes, spleen, and liver starting from the 1st day after the colonization of germ-free piglets with the SF 1591 strain. From Fig. 1, it is apparent that maximal counts were found 3 to 6 days after the association, followed by a slow decline during the next weeks. The peak of bacterial counts correlated with a transient elevation of body temperature.

No bacteria could be detected in the blood and organs of piglets colonized with the mR 595 strain, and bacteria were found in the mesenteric lymph nodes only occasionally (data not shown). No fever was registered in these piglets. All animals appeared to be normal, as was their food intake.

Protective effect of colonization of GF piglets with a rough *Salmonella* strain. To study the immune response and protective effect of colonization with a rough *Salmonella* strain, two groups of 1-week-old GF piglets consisting of seven and six animals were perorally associated with strains SF 1591 and mR 595, respectively. Two piglets from each group were sacrificed 1 week later for bacterial counting (Table 1) and serum collection. The difference between the two groups was striking.

Appreciable numbers of bacteria were found in the mesenteric lymph nodes, spleen, liver, and blood of piglets associated with strain SF 1591 (chemotype Ra).

On the other hand, in the second group associated with strain mR 595 (chemotype Re), the mesenteric lymph nodes of only one piglet contained bacteria.

The remaining piglets associated with rough mutants were subsequently infected with streptomycin-resistant *S. typhimurium* LT2, and streptomycin was applied in a milk diet. Antigenic change of intestinal bacteria was completed within 1 day and remained constant, as revealed by slide agglutination. All five piglets colonized previously with strain SF 1591 survived and were sacrificed 1 week later for experimental reasons.

TABLE 3. Bacterial counts in blood, spleen, liver, and mesenteric lymph nodes of piglets infected with strain mR 595 (Rc) and reinfected with virulent smooth strain *S. typhimurium* LT2

Tissue or fluid	Bacterial count[a] in piglet:			
	2/681	0/688	1/688	3/688
Blood	Positive	6.7×10^3	8.0×10^2	1.9×10^2
Spleen	Positive	5.5×10^5	3.6×10^4	7.2×10^3
Liver	Positive	2.0×10^5	1.6×10^4	2.8×10^3
MLN[b]	Positive	8.1×10^6	8.2×10^5	1.8×10^5
Clinical state (day)	Dead (2)	Dead (3)	Ante finem (6)	III (8)

[a] Values represent counts per gram of tissue or milliliter of fluid.
[b] MLN, mesenteric lymph nodes.

TABLE 4. Antibody responses of GF piglets against smooth and rough LPSs 1 week after infection with rough *Salmonella* strains

LPS	Antibody responses[a] of piglets infected with strain:	
	SF 1591 (Ra)	mR 595 (Re)
LT2 (S)	100, 100, 100, 100, 100, 0	10, 0, 0, 0, 0, 0
R4 (Ra)	100, 10, 10, 10, 10, 0	10, 5, 0, 0, 0, 0
R595 (Re)	100, 100, 100, 100, 10, 0	10, 0, 0, 0, 0, 0

[a] The titer determined by ELISA is expressed as the reciprocal of the dilution. A value of 0 means a negative reaction at a dilution of 1:5.

In contrast to this, three of four piglets colonized with strain mR 595 died in a septic state within 1 week and the fourth was sacrificed in the terminal stage of *Salmonella* infection. Bacterial counts in mesenteric lymph nodes and organs are shown in Tables 2 and 3.

The majority of bacteria detected in the mesenteric lymph nodes and other organs of piglets in the former group corresponded to the Ra chemotype surviving from the 1st week, in contrast to the latter group, in which only smooth bacteria were found.

Antibody response against LPS in GF piglets colonized with *Salmonella* strains. The antibody response against the three LPSs used could be detected in most of the piglets colonized with strain SF 1591 beginning on day 3 after colonization, and all but one were positive after 1 week. In contrast, only two piglets of the six colonized with strain mR 595 had low titers after 1 week (Table 4).

The results of examination of sera from piglets surviving subsequent infection with the virulent strain *S. typhimurium* LT2 are shown in Table 5. It is apparent that this change of the intestinal microbial population did not induce the change in serum antibody titers. Surprisingly, most sera reacted with all three of the LPSs tested. The majority of serum antibodies belonged to the IgM isotype.

The antibodies against LPS were not detected in intestinal washings obtained 1 week after colonization with rough mutants, but they were detected 1 week after subsequent infection with the smooth virulent strain (Table 6). In contrast to the results obtained with sera, the reactivity with S LPS was lacking in intestinal washings and the highest titers were detected with Ra LPS. IgA was found in intestinal washings to be absorbed on the surfaces of intestinal bacteria in all piglets colonized with the SF 1591 mutant and subsequently infected with the virulent LT2 strain but not in piglets colonized with the mR 595 mutant.

The cross-reactivity of serum antibodies was analyzed by the Western blotting technique. LPSs were subjected to SDS-polyacrylamide gel electrophoresis, and fractions were transferred to a nitrocellulose membrane. Unbound sites were blocked with skim milk, and individual lanes were treated with

TABLE 5. Antibody responses against smooth and rough LPSs of GF piglets infected with rough *Salmonella* strains 1 week after reinfection with smooth *S. typhimurium* LT2

LPS	Antibody responses[a] of piglets infected with an R strain and reinfected with an S strain:	
	SF 1591 (Ra)+ LT2 (S)	mR 595 (Re)+ LT2 (S)
LT2 (S)	100, 1,000, 10, 10, 100	100, 100
R4 (Ra)	10, 100, 10, 10, 10	100, 10
R595 (Re)	100, 100, 100, 10, 100	100, 100

[a] The titer determined by ELISA is expressed as the reciprocal of the dilution.

TABLE 6. Antibody concentrations in intestinal washings of GF piglets infected with rough *Salmonella* strains 1 week after reinfection with smooth *S. typhimurium* LT2

LPS	Antibody responses[a] of piglets infected with an R strain and reinfected with an S strain:	
	SF 1591 (Ra)+ LT2 (S)	mR 595 (Re)+ LT2 (S)
LT2 (S)	0, 0, 5, 0, 0	0
R4 (Ra)	100, 200, 200, 10, 5	10
R595 (Re)	0, 0, 5, 10, 10	10

[a] The titer is expressed as the reciprocal of the dilution. A value of 0 means a negative reaction at a dilution of 1:5.

either hyperimmune serum or the sera of individual piglets. A typical result obtained with LT2 LPS is shown in Fig. 2. A broad spectrum of fractions of different molecular masses (with a ladder of oligomers and polymers characteristic of the LPSs of smooth strains) was obtained with hyperimmune rabbit antiserum. On the other hand, all piglet sera reacted only with the low-molecular-weight fraction near the front representing the unsubstituted core (8, 36).

Rough LPS analyzed by the Western blotting technique provided a similar picture (data not shown). Therefore, the LT2 LPS originally prepared by the phenol-water method was re-extracted with PCP to remove the "rough" portion of the smooth LPS. The purified "smooth" fraction insoluble in PCP was then tested in the ELISA and Western blot systems, and it was found that both reactivities were lost (data not shown).

DISCUSSION

Mechanisms enabling intestinal microorganisms to cross the mucosa of the gut, thus leading to the appearance of bacteria in the mesenteric lymph nodes, spleen, and liver or elsewhere in the body, are more or less deliberately designated in terms of invasion, penetration, and translocation (2, 14, 34). However, leaving apart the question of which of these expressions is the most suitable, it should be kept in mind that it is, in fact, a multistep process including such different events as adhesion to the mucus layer and penetration of it and interaction with enterocytes (13, 20) and/or the M-cell plasma membrane (3, 21), leading to endocytosis, intracellular survival, or even multiplication and active movement or passive transport to other parts of the body. Individual steps are controlled by different mechanisms, and it is very difficult to analyze them and define their roles under in vivo conditions.

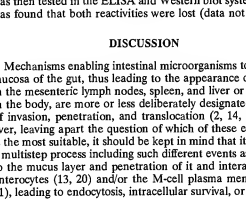

FIG. 2. Western blot analysis of interaction of the LPS of the smooth strain *S. typhimurium* LT2 with either hyperimmune rabbit antiserum and/or sera of piglets colonized for 1 week with the rough strain *S. typhimurium* SF 1591 followed by peroral infection with *S. typhimurium* LT2. Lanes: A, hyperimmune rabbit antiserum; B to E, sera of individual piglets.

No differences in the abilities of our three *Salmonella* strains, LT2 (S), SF 1591 (Ra), and mR 595 (Re), to colonize the intestinal tracts of GF piglets were seen. This is certainly due to the absence of microbial flora in the gut (43). However, striking differences were found in the abilities to penetrate the mucosa (translocate) and spread into the organs of these animals. In contrast to the virulent smooth strain *S. typhimurium* LT2, which penetrates and multiplies freely, leading to death within 1 or 2 days, the deep-rough mutant *S. minnesota* mR 595 only rarely appeared in mesenteric lymph nodes of colonized GF piglets during the whole time of observation. Therefore, it is highly probable that the inhibition of spreading in this case is independent of specific immunity. This result may be relevant to the finding that the highly LPS-deficient mutant strain *S. typhimurium* SF 5325 (chemotype Rb_2) is less able to penetrate the mucus and adhere to the epithelial cell membrane than is the avirulent smooth strain (25). From our results, it seems that *S. typhimurium* SF 1591 behaves more like the avirulent smooth strain used in the studies mentioned above. It readily penetrates the epithelial layer and spreads over the body. However, this process is self-limited, as is apparent from Fig. 1. Still more, such a curve is highly likely to be the result of development of specific systemic and local immunity, leading not only to progressive elimination of bacteria from organs and mesenteric lymph nodes but also to inhibition of further penetration of the body by intestinal bacteria (39, 40).

There are also controversial data about the protective effect of hyperimmune sera on the course of experimental *Salmonella* infection (28). A positive effect was usually obtained only when both the challenge organism and serum or antibodies were applied intraperitoneally (4, 6, 12). By using different mouse strains of the same C3H lineage, it has been shown that the protective effect of hyperimmune sera is highly dependent on the degree of inherited resistance of a given strain of mice (12).

It has been shown that peroral application of hyperimmune serum against *E. coli* O55 (highly pathogenic for GF piglets) prevents death from otherwise 100% lethal septicemia (37) in spite of the fact that bacteria were detected in mesenteric lymph nodes and other organs (10). On the other hand, the similar treatment of GF piglets with hyperimmune sera against *S. typhimurium* influenced neither penetration nor lethality. This can be correlated with local cellular changes and cytokine production in the intestine (42). The application of hyperimmune serum did not influence the translocation of nonpathogenic *E. coli* O86 in GF piglets which was also seen in conventional piglets suckling antibody-containing colostrum (40). These data correlate with those obtained with mice (2). In summary, it seems that conventional serum or colostrum does not play an important role in the control of penetration by bacteria from the intestinal tract. On the other hand, implanted hybridomas producing secretory IgA antibodies against the smooth LPS epitope of *S. typhimurium* induced protection against peroral infection with a virulent smooth strain of *S. typhimurium* (33).

There is general agreement that live attenuated *Salmonella* vaccines are more effective in inducing protective immunity than are killed bacteria, especially when administered perorally (5, 18). This is usually explained by the fact that only viable vaccines induce both cellular and humoral responses, including a local IgA response, in contrast to dead vaccines, which preferentially stimulate humoral immunity, especially when applied parenterally (5, 18). Differences in gut responses

to isolated antigens and dead and live vaccines were recently documented and discussed (32).

It is usually stressed that only vaccines containing smooth-type LPS are effective against infections caused by pathogenic gram-negative bacteria (18). On the other hand, there is evidence that rough bacteria may also induce protective immunity against pathogenic smooth strains (17, 23). It is likely that cross-reactive antibodies are formed which interact with both rough and smooth bacteria and/or their LPSs and thus protect against an endotoxic effect (9, 31, 35, 45). Some of them are directed against epitopes accessible in rough, as well as in smooth, LPS with a complete O-antigenic determinant (8, 9), indicating that at least some rough epitopes are accessible in the complete smooth LPS. As shown in Fig. 1, the serum antibodies formed by piglets either colonized with strain SF 1591 alone or subsequently infected with the LT2 smooth strain reacted only with the low-molecular-weight fraction of both the smooth and rough LPSs. The reason why piglets infected for 1 week with a smooth strain of *S. typhimurium* do not form antibodies specific for epitopes of the smooth LPS is not clear. We may only speculate that the penetration of the smooth strain was very restricted as a result of a previous immune response to the rough strain, resulting in negligible penetration of the mesenteric lymph nodes and spleen by the smooth strain.

The absence of antibodies against smooth LPS in the intestinal washings of piglets surviving infection with the virulent strain *S. typhimurium* LT2 (shown in Table 6) is surprising. However, it has been shown that the bacteria in the intestinal contents are coated with IgA (42). We cannot exclude the possibility that specific IgA antibodies against epitopes of the smooth LPS were absorbed by the intestinal microbial population. On the other hand, it is also possible that the protective effect of previous colonization of GF piglets with rough strain SF 1591 could not be ascribed solely to the immune response against some rough or smooth epitopes of the LPS. Recently, a number of surface *Salmonella* antigens were shown to play an important role not only in virulence and invasion (13, 15, 20) but also in the protective immune response (19, 22, 30). Because some of them are expressed only under stress conditions which also occur during natural infection and because the possibility of their production by rough strains cannot be excluded, their role in the protective effect needs to be elucidated.

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